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(54) Title: DETERGENT CONTAINING PROTEASE AND INHIBITOR AND NOVEL INHIBITORS FOR USE THEREIN (57) Abstract A detergent composition and additive comprising a protease and a reversible protease inhibitor of the peptide or protein type, wherein the ratio of the dissociation constant to the protease concentration in the range from 0.006 to 6. When the protease is subtilisin, the protease inhibitor is preferably a modified subtilisin inhibitor of Family VI.		

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DETERGENT CONTAINING PROTEASE AND INHIBITOR AND NOVEL INHIBITORS FOR USE THEREIN

TECHNICAL FIELD

This invention relates to an improved detergent composition comprising a protease (particularly a subtilisin) and a reversible protease inhibitor of peptide or protein type, to a detergent additive comprising such a protease and inhibitor and to a method for stabilizing a protease.

The invention also relates to novel modified subtilisin inhibitors for use in said detergent, to a recombinant DNA molecule comprising a nucleotide sequence coding for the modified subtilisin inhibitor, to a transformed host organism comprising the DNA and to a method of producing the modified inhibitor.

BACKGROUND ART

Proteases, especially subtilisins, are widely used as ingredients in commercial detergents. A major problem in formulating protease-containing detergents, especially liquid detergents, is that of ensuring enzyme stability during storage.

The prior art has dealt extensively with improving the storage stability. As an example, JP-A 62-269689 demonstrates improvement of the stability of a protease (e.g. a subtilisin) in a liquid detergent by incorporation of a protease inhibitor of protein type. As stated in said publication, the protease inhibitor should ideally show essentially no inhibiting effect under dilute washing conditions, i.e. when the detergent is in use.

STATEMENT OF THE INVENTION

We have found that in the known detergents containing protease and inhibitor, the protease is almost totally inhibited under dilute washing conditions. We have also found that by a suitable choice of inhibitor for a given protease, it is possible to essentially avoid inhibition at the dilute conditions of washing, while still achieving effective enzyme stabilization in the detergent during storage.

We have also found that subtilisin inhibitors with this improved performance can be derived from known inhibitors by substituting certain amino acids. The novel inhibitors can be produced by known genetic engineering methods.

Accordingly, the invention provides a detergent composition comprising a protease and a reversible protease inhibitor of peptide or protein type, characterized in that the ratio of the dissociation constant to the protease concentration is in the range from 0.006 to 6, or that the dissociation constant is in the range from 0.05 to 50 μM . The invention also provides a detergent additive comprising protease in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of peptide or protein type having an dissociation constant in the above range. Further, the invention provides a method for stabilizing a protease by incorporation of a protease inhibitor as described.

Another aspect of the invention provides a detergent composition and a detergent additive comprising a subtilisin, characterized by further comprising a modified subtilisin inhibitor of Family VI having one or more of the following amino acid substitutions at the indicated position:

P6: Ala, Glu or Lys,
P5: Gly, Val, Leu or Pro
P4: Val, Pro, Trp, Ser, Glu or Arg,
P3: Trp, Glu, Ala, Arg, Pro, Ser, Lys or Trp,
5 P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala,
P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys or Ala,
P'1: Gln, Ser, Thr, Ile, Lys, or Pro,
P'2: Val, Glu, Arg, Pro or Trp,
P'3: Glu, Gln, Asn, Val, Phe or Tyr.

10 The invention also provides a modified subtilisin inhibitor of family VI,
as defined above, excluding:

Eglin B and C substituted with
Ser or Pro at position 44 (P2),
Leu, Arg, Phe, Tyr at 45 (P1) or
15 Glu, Ser or Thr at 46 (P'1),
Eglin C substituted with Arg45, Ser46 and
CI-2 substituted with Tyr, Ala or Lys at 59 (P1).

Further, the invention provides a recombinant DNA molecule
comprising a nucleotide sequence coding for a modified subtilisin inhibitor as
20 defined above, a transformed host organism comprising this DNA and a method
of producing the modified inhibitor comprising cultivation of this transformed host
organism.

Modified subtilisin inhibitors of family VI are known (EP 332,576, C.
Langstaff et al., *Biochemistry*, 1990, 29, 7339-7347), but their use in detergents
25 and the resulting advantages have not been disclosed or suggested.

DETAILED DESCRIPTION OF THE INVENTION

Protease

The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant subtilisins such as those described in WO 89/06279 and DK 0541/90. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μM , especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l) as pure enzyme protein.

Inhibitor

According to the invention, the inhibitor is chosen for a given detergent (protease type and concentration etc.) so that the dissociation constant (K_D) is high enough to allow adequate release of protease when the detergent is diluted with water, yet the dissociation constant is low enough to allow efficient inhibition in the concentrated detergent during storage. K_D is commonly defined for a given protease and a given inhibitor in a given system as the equilibrium constant

$$K_D = [E] * [I] / [EI]$$

where the square brackets indicate molar concentration of free enzyme (E), free inhibitor (I) and enzyme-inhibitor complex (EI), respectively.

The ratio of the dissociation constant to the protease concentration is preferably from 0.06 to 6. The dissociation constant is preferably from 1 to 10 μM (i.e. 10^{-6} - 10^{-5} M).

The desired K_D is achieved by suitable selection of protease and inhibitor. The inhibitor may be one of the novel modified inhibitors provided by the invention, or it may be selected from among the many known inhibitors, e.g. *Streptomyces* subtilisin inhibitor used together with trypsin. See e.g. Lakowski, Jr. & Kato, Ann. Rev. Biochem. 49:593-626 (1980) and S. Murao et al., in Protein Protease Inhibitor - The Case of Streptomyces Subtilisin Inhibitor (1985) at pp. 1-14 for a general description of known inhibitors.

The amount of inhibitor is preferably such that the molar ratio of inhibitor reactive site to protease active site is above 0.6, preferably 1-10.

10 Novel inhibitor

The novel inhibitors provided by the invention are derived from the known inhibitors of Family VI, described in the above references, e.g. from barley subtilisin inhibitor CI-1 or CI-2, potato subtilisin inhibitor (PSI) Eglin B or C, tomato subtilisin inhibitor or *Vicia* subtilisin inhibitor (VSI).

15 Inhibitors of this family are known to strongly inhibit the subtilisins commonly used in detergents, with inhibitor dissociation constants generally below 10^{-10} M. We have found that by using these inhibitors to stabilize a protease in a detergent, the protease is so strongly bound that very little protease activity is released when the detergent is diluted for use in washing, and the
20 protease remains almost completely inactive. We have therefore realized a need for a modified inhibitor with weaker binding to the protease.

The following shows a comparison of the sequences in the binding region of some family VI inhibitors. Starting from the reactive site, amino acid positions are numbered P1, P2 etc. in the direction of the N-terminal and P'1, P'2
25 etc towards the C-terminal.

<u>Inhibitor</u>	<u>P6</u>	<u>P5</u>	<u>P4</u>	<u>P3</u>	<u>P2</u>	<u>P1</u>	<u>P'1</u>	<u>P'2</u>	<u>P'3</u>
CI-1	Asp	Ala	Met	Val	His	Leu	Asn	Phe	Asp
CI-2	Gly	Thr	Ile	Val	Thr	Met	Glu	Tyr	Arg
PSI	Gly	Ser	Pro	Val	Thr	Met	Asp	Phe	Arg
5 Eglin C	Gly	Ser	Pro	Val	Thr	Leu	Asp	Leu	Arg
Eglin B	Gly	Ser	Pro	Val	Thr	Leu	Asp	Leu	Arg
TSL-1	Gly	Ser	Pro	Ile	Thr	Leu	Asp	Tyr	Leu
VSI	Gly	Ser	Phe	Val	Thr	Ala	Asp	Tyr	Lys

Variation

10	Asp	Ala	Met	Val	His	Leu	Asn	Phe	Asp
	Gly	Thr	Ile	Ile	Thr	Met	Glu	Tyr	Arg
		Ser	Pro			Ala	Asp	Leu	Leu
			Phe						Lys

Modifications according to invention

15	Ala	Gly	Val	Tyr	Ser	Arg	Gln	Val	Glu
	Glu	Val	Pro	Glu	Lys	Glu	Ser	Glu	Gln
	Lys	Leu	Trp	Ala	Arg	Val	Pro	Arg	Asn
		Pro	Ser	Arg	Pro	Ser	Ile	Pro	Val
			Glu	Pro	Glu	Lys	Thr	Trp	Phe
20			Arg	Ser	Val	Tyr	Lys		Tyr
				Lys	Tyr	Pro			
				Trp	Trp	Ala			
					Ala	Trp			

It appears that the inhibitors show a marked homology in the binding 25 region. We have now found that the protease-inhibitor binding can be suitably weakened by substituting one or more of these amino acids, e.g. with one that is not represented at that position, i.e. with one that has a different side chain length and/or is differently charged from those represented. The modified inhibitors are resistant to hydrolysis by the protease.

A preferred inhibitor is Cl-2 substituted with Arg, Pro or Glu at position P3, Lys or Arg at P2, and/or Glu, Arg or Pro at P1. Another preferred inhibitor is PSI substituted with Tyr at P3, Lys or Arg at P2, Arg, Tyr or His at P1 and/or Trp at P'1.

5 The novel inhibitors may be produced by known genetic engineering techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-
10 directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

15 The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression
20 plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA *in vivo*. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent on the type of host strain used. The expression of the mutant inhibitor may be done intracellularly or extracellularly. In the latter case, the DNA sequence coding
25 for the mutant inhibitor is fused in frame to a DNA sequence encoding a suitable peptide signalling secretion. The secretion signal should preferably be cleaved off *in vivo*, resulting in secretion of the mature mutant inhibitor into the growth medium.

 Various species of Bacilli, including *Bacillus alkalophilus*, *B.*
30 *amyloliquefaciens*, *B. brevis*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. stearothermophilus*, and *B. subtilis*, are known to secrete proteins efficiently. In many cases this has also been shown to be the case for heterologous proteins.

Since expression of a secreted protease inhibitor has the potential advantage of facilitating purification, it is obviously interesting to attempt to express the inhibitor as a secreted product from a *Bacillus* strain. This could for instance be accomplished by combining the structural part of the inhibitor with the promoter
5 and signal peptide of a well expressed and secreted *Bacillus* enzyme as for instance the maltogenic amylase from *B. stearothermophilus* (Diderichsen, B. and Christiansen, L. Cloning of a maltogenic alpha-amylase from *Bacillus stearothermophilus*, FEMS Microbiol. Lett. 56:53-60. 1988) or the alpha- amylase from *B. licheniformis* (Jørgensen, P.L., C.K. Hansen, G.B. Poulsen and B. Diderichsen. *In vivo* genetic engineering: Homologous recombination as a tool for plasmid
10 construction, GENE 96: 37-41, 1990). This may be accomplished in many ways as known by people skilled in the art. One way is to use *in vivo* genetic engineering (Jørgensen et al. 1990, op. cit.). The advantage of this method is that it easily generates a perfect fusion between signal peptide and mature inhibitor
15 which according to well documented rules for signal peptide processing would be expected to give the correct N-terminal amino acid residue of the inhibitor.

In one method of producing barley CI-2A inhibitor and variants hereof, a filamentous fungus is used as the host organism. The filamentous fungus host organism may conveniently be one which has previously been used as a host for
20 producing recombinant proteins, e.g. a strain of *Aspergillus* sp., such as *A. niger*, *A. nidulans* or *A. oryzae*. The use of *A. oryzae* in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

For expression of CI-2A inhibitor and variants in *Aspergillus*, the DNA sequence encoding the protease inhibitor is preceded by a promoter. The
25 promoter may be any DNA sequence exhibiting a strong transcriptional activity in *Aspergillus* and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene
30 encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral alfa-amylase, *A. niger* acid stable alfa-amylase, *A. niger*

glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease or *A. oryzae* triose phosphate isomerase.

In particular when the host organism is *A. oryzae*, a preferred promoter for use in the process of the present invention is the *A. oryzae* TAKA
5 amylase promoter as it exhibits a strong transcriptional activity in *A. oryzae*. The sequence of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

To ensure secretion of the inhibitor or variants hereof from the host
10 cell, the DNA sequence encoding the inhibitor may be preceded by a signal sequence which may be a naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an
Aspergillus sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei*
15 lipase or proteinase, or a gene encoding a *Humicola* cellulase, xylanase or lipase.

Detergent

The detergent of the invention may be in any convenient form, e.g. powder, granules or liquid. A liquid detergent may be aqueous, typically containing 20-70% water and 0-20% organic solvent.

20 The detergent comprises surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 5-30% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alcohol ethoxy sulphate (AES) or soap. It may also contain 3-20% anionic surfactant such as nonyl phenol ethoxylate or alcohol
25 ethoxylate.

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-10. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, or it may be unbuild (i.e. essentially free of a detergent builder). It may also contain
30 other conventional detergent ingredients, e.g. fabric conditioners, foam boosters, bactericides, optical brighteners and perfumes.

Specific examples of detergents according to the invention may be obtained from the compositions disclosed in WO 89/04361, DK 5111/89 or PCT/DK91/00243 by incorporating protease and inhibitor according to the invention. PCT/DK91/00243 is incorporated herein by reference.

5 The invention is particularly applicable to the formulation of liquid detergents with pronounced enzyme stability problems, e.g. those containing oxidizing agents. Such detergents typically contain 1-40%, especially 5-20% oxidizing agent. They may be granular detergents containing granules of a perborate or percarbonate and separate granules containing enzyme and
10 inhibitor according to the invention, or they may be aqueous or non-aqueous liquid detergents containing hydrogen peroxide, a perborate or a percarbonate (see e.g. EP 378,261, EP 378,262, EP 294,904, EP 368,575).

Detergent additive

The protease and inhibitor may be included in the detergent of the
15 invention by separate addition or by adding the combined additive provided by the invention. The additive will usually contain 0.2-8 mM protease (0.5-20%) and have an inhibitor/protease ratio as described above.

The detergent additive may be in liquid form for incorporation in a liquid detergent. A liquid additive may contain 20-90% propylene glycol; 0.5-3%
20 (as Ca) of a soluble calcium salt; 0-10% glycerol; minor amounts of short-chain fatty acids and carbohydrate; and water up to 100%.

EXAMPLE 1

Expression of barley subtilisin inhibitor CI-2A in *Saccharomyces cerevisiae*

The barley subtilisin inhibitor and variants hereof according to the
25 invention can be produced biosynthetically in a yeast host expressing a DNA sequence encoding the inhibitor.

To achieve secretion to the growth medium, the DNA sequence encoding the inhibitor can be fused to another DNA-sequence encoding a signal

peptide functional in yeast. An example hereof is the *Saccharomyces cerevisiae* MF-alfa-1 leader sequence (Kurjan & Herskowitz, Cell 30, 933-943 (1982). A preferred construction uses the DNA sequence encoding the entire 85 aminoacid MF-alfa-1 leader sequence including the dibasic site LysArg. In that way, an efficient secretion of CI-2A inhibitor with the correct N-terminal is achieved.

Plasmid construction

All expression plasmids are of the C-POT type. Such plasmids are described in EP patent application No. 85303702.6 and are characterized in containing the *S. pombe* triose phosphate isomerase gene (POT) for the purpose of plasmid stabilization. A plasmid containing the POT-gene is available from a deposited *E.coli* strain (ATCC 39685). The plasmids furthermore contain the *S. cerevisiae* triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pLaC200 described in the patent application WO 89/02463, except for the region defined by the EcoRI/XbaI restriction fragment encoding a signal/leader/insulin precursor sequence. In this application, the region is replaced by a fragment encoding MF-alfa-1 leader fused to the inhibitor sequence. The sequence of the fragment is shown in Sequence Listing ID No. 1 (P1 is located at Met 59). The isolation of the barley CI-2A subtilisin inhibitor cDNA is described by Williamson et al. Eur. J. Biochem. 165, 99-106 (1987). Cloning of the MF-alfa-1 leader is described by Kurjan & Herskowitz (reference given above). Modifications and assembly of the two sequences were carried out using entirely standard techniques. In particular, the KpnI and ClaI restriction sites at positions 495 and 519, respectively, were generated by introducing silent mutations into the inhibitor gene. This was done carrying out *in vitro* mutagenesis on the inhibitor gene. A map of the expression plasmid pYAC12 is shown in Figure 2.

Introduction of mutations into the inhibitor gene

Mutant CI-2A genes were generated using PCR mutagenesis, which was carried out as follows: A primer carrying the mutation flanked by homologous sequences and carrying the introduced KpnI-site was used together

with another primer homologous to sequences in the T_{TPI} region in a PCR amplification reaction. In that way, fragments were generated, which contained the desired mutations. The ends were trimmed with the restriction enzymes KpnI and XbaI, purified on agarose gels, and cloned into pYAC12 previously digested with the same restriction enzymes. The presence of the mutation was verified by DNA sequencing. The primers used are listed below.

<u>Mutation</u>	<u>Primer sequence</u>
P4 Ile -> Pro	5'-CCGGTGGGTACCCCAGTGACCATGGAA-3'
P4 Ile -> Trp	5'-CCGGTGGGTACCTGGGTGACCATGGAA-3'
10 P3 Val -> Glu	5'-GTGGGTACCATTGAAACCATGGAATAT-3'
P3 Val -> Ala	5'-GTGGGTACCATTGCTACCATGGAATAT-3'
P3 Val -> Arg	5'-GTGGGTACCATTAGAACCATGGAATAT-3'
P3 Val -> Tyr	5'-GTGGGTACCATTTACACCATGGAATAT-3'
P3 Val -> Pro	5'-GTGGGTACCATTCCAACCATGAAGTAT-3'
15 P2 Thr -> Glu	5'-GTGGGTACCATTGTGGAAATGGAATATCGG-3'
P2 Thr -> Val	5'-GTGGGTACCATTGTGGTTATGGAATATCGG-3'
P2 Thr -> Arg	5'-GTGGGTACCATTGTGAGAATGGAATATCGG-3'
P2 Thr -> Tyr	5'-GTGGGTACCATTGTGTACATGGAATATCGG-3'
P2 Thr -> Pro	5'-GTGGGTACCATTGTGCCAATGGAATATCGG-3'
20 P1 Met -> Glu	5'-GTGGGTACCATTGTGACCGAAGAATATCGGATC-3'
P1 Met -> Val	5'-GTGGGTACCATTGTGACCGTTGAATATCGGATC-3'
P1 Met -> Arg	5'-GTGGGTACCATTGTGACCAGAGAATATCGGATC-3'
P1 Met -> Tyr	5'-GTGGGTACCATTGTGACCTACGAATATCGGATC-3'
P1 Met -> Pro	5'-GTGGGTACCATTGTGACCCCAGAATATCGGATC-3'

25 The sequence of the other primer used for generation of PCR products, and which has homology to the T_{TPI} terminator region is: 5'-TTAAGTGGCTCAGAATG-3'

Expression of mutant CI-2A inhibitors in yeast

Plasmids prepared as described above were transformed into a *S. cerevisiae* strain carrying deletions in the TPI gene by selecting for growth on glucose.

- 5 The transformed yeast strains were grown on YPD medium (Sherman, F. et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory 1981). 100 ml medium in shake-flasks was inoculated with individual transformants and shaken at 30°C for approx. 48 hours after which the inhibitor could be purified from the medium.

10 **EXAMPLE 2**Expression of barley chymotrypsin inhibitor CI-2A in *Aspergillus oryzae*Plasmid constructions

Cloning and expression of *Humicola lanuginosa* lipase in *Aspergillus oryzae* is described in EP 305,216. The same host/vector system can be used for
15 expression and secretion of barley chymotrypsin inhibitor CI-2A. The lipase expression plasmid is termed p960 and makes use of the *A. oryzae* TAKA amylase promoter for driving the transcription and the *Aspergillus niger* glucoamylase transcription terminator.

The plasmid p960 was slightly modified in order to obtain a vector for
20 cloning the inhibitor gene. p960 was digested with NruI and BamHI restriction enzymes. Between these two sites the BamHI/NheI fragment from pBR322, in which the NheI-site was filled in with Klenow polymerase and dNTP's, was cloned, thereby creating plasmid pAO1 (Fig. 3) which contains unique BamHI and NheI sites facilitating cloning of BamHI/XbaI fragments.

- 25 A BamHI/AvaI linker with the sequence

BamHI

GATCCACCATGAGGAGCTCCCTTGTGCTGTTCTTTGTCTCTGCGTGGACGGCCTTGGCCAGTC
 GTGGTACTCCTCGAGGGAACACGACAAGAAACAGAGACGCACCTGCCGGAACCGGTCAG
 MetArgSerSerLeuValLeuPhePheValSerAlaTrpThrAlaLeuAlaSerP

5

CTATTCGTCGAAGCTCAGTGGAGAAGAAGC AvaI

GATAAGCAGCTTCGAGTCACCTCTTCTTCGGGCT

roIleArgArgSerSerValGluLysLysPro

10

encoding the *Humicola lanuginosa* lipase pre-pro sequence and part of the CI-2A inhibitor was combined with the Aval/XbaI fragment from pYACI2 and cloned into pAO1 digested with BamHI and NheI, thereby creating the expression plasmid pAHLCI2 (Fig. 4). The sequence of the BamHI/XbaI insert is shown in Sequence 15 Listing ID No. 3 (P1 at Met 59).

Transformation of *Aspergillus oryzae* (general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) was inoculated with spores of *A. oryzae* and incubated with shaking for about 24 hours. The mycelium was harvested by filtration through miracloth and washed with 200 ml of 0.6 M MgSO₄. The mycelium was suspended in 15 ml of 1.2 M MgSO₄, 10 mM NaH₂PO₄, pH = 5.8. The suspension was cooled on ice and 1 ml of buffer containing 120 mg of Novozym[®] 234, batch 1687 was added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) was added and incubation with gentle agitation continued for 1.5 - 2.5 hours at 37°C until a large number of protoplasts was visible in a sample inspected under the microscope.

The suspension was filtered through miracloth, the filtrate transferred to a sterile tube and overlaid with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation was performed for 15 min. at 1000 g and the protoplasts were collected from the top of the MgSO₄ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM CaCl₂) were added to the protoplast suspension and the mixture was centrifugated for 5 min. at 1000 g. The

protoplast pellet was resuspended in 3 ml of STC and repelleted. This was repeated. Finally, the protoplasts were resuspended in 0.2 - 1 ml of STC.

100 μ l of protoplast suspension was mixed with 5 - 25 μ g of p3SR2 (an *A. nidulans* amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) in 10 μ l of STC. The mixture was left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM CaCl_2 and 10 mM Tris-HCl, pH = 7.5 was added and carefully mixed (twice) and finally 0.85 ml of the same solution was added and carefully mixed. The mixture was left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet was resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts were spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4 - 7 days at 37°C spores were picked, suspended in sterile water and spread for single colonies. This procedure was repeated and spores of a single colony after the second reisolation were stored as a defined transformant.

Expression of the barley inhibitor CI-2A in *A. oryzae*

pAHLCl2 was transformed into *A. oryzae* IFO 4177 by cotransformation with p3SR2 containing the amdS gene from *A. nidulans* as described above. Protoplasts prepared as described were incubated with a mixture of equal amounts of pAHLCl2 and p3SR2, approximately 5 μ g of each were used. 9 transformants which could use acetamide as sole nitrogen source were reisolated twice. After growth on YPD for three days, culture supernatants were analyzed for inhibitor activity.

EXAMPLE 3

Purification of the wild-type CI-2 inhibitor and mutants thereof

Fermentation broths containing either the wild-type CI-2 inhibitor or one of the following CI-2 inhibitor mutants: CI-2(M59P), CI-2(V57E), CI-2(M59E),

CI-2(M59R), CI-2(V57R) and CI-2(V57P+E60K), produced as described in example 1, were filtered on a pressure filter (Zeitz K 250-Neu) provided with 0.5% filter aid, and subsequently on a Zeitz EK-1 filter provided with 0.5% filter aid. The filtrate was diluted to a conductivity of <4 mS with 50 mM sodium acetate, pH 4.4, and water, and the pH was adjusted to 4.4. The filtrate was then subjected to chromatography on a S-Sepharose FPLC column using a 50 mM sodium acetate buffer, pH 4.4, and a 50 mM sodium acetate buffer supplemented with 1M NaCl. Elution of the column was performed with a 0-100% gradient of 50 mM sodium acetate buffer supplemented with 1M NaCl.

10 To effect a change of buffer, the eluate was run through a Sephadex G-25 column into two different buffers: (a) 50 mM glycine-NaOH buffer, pH 9.6, and (b) 50 mM H₃BO₃-NaOH buffer, pH 10.2 (buffer (b) being used for basic mutants (CI-2(M59R), CI-2(V57R) and CI-2(V57P+E60K)). The eluate was then subjected to chromatography on a Q-Sepharose column using either buffer (a) or
15 buffer (b), as appropriate. The column was eluted with a gradient of 0-1 M NaCl. Inhibitor-containing fractions were collected and used in the subsequent experiments.

EXAMPLE 4

Interaction of protease with inhibitor

20 The interaction of Alcalase® with wild-type CI-2, CI-2(M59P) and CI-2(V57E), respectively, was studied in a 0.1 M Tris-HCl buffer, pH 8.6, at 25°C, using the synthetic peptide substrates Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Ala-pNA (both available from Sigma) to determine residual activity after reacting the protease with the inhibitor in amounts from 0 to 1.5 times the protease
25 concentration.

The following dissociation constants were determined using non-linear regression essentially as described in M. Tashiro et al., Agric. Biol. Chem. 55(1), 1991, pp. 265-267.

Inhibitor	Dissociation constant (K_d)
Wild-type CI-2	4×10^{-12} M
CI-2(M59P) (mutated in P1)	5×10^{-9} M
5 CI-2(V57E) (mutated in P3)	1×10^{-10} M

The results show that it is possible to change the dissociation constant by several orders of magnitude by single amino acid substitutions in the binding region of the inhibitor.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Novo Nordisk A/S
- (ii) TITLE OF INVENTION: Detergent containing protease and inhibitor and novel inhibitor
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Novo Nordisk A/S
 - (B) STREET: Novo Alle
 - (C) CITY: Bagsvaerd
 - (E) COUNTRY: Denmark
 - (F) ZIP: 2880
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Thalsoe-Madsen, Birgit
 - (C) REFERENCE/DOCKET NUMBER: 3486.204-WO
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: +4544448888
 - (B) TELEFAX: +4544493256
 - (C) TELEX: 37304

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 592 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: barley
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 77..580

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 77..331

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 332..580

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATAAAGGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA	109
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu	
-85 -80 -75	
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA	157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu	
-70 -65 -60	
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT	205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp	
-55 -50 -45	
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA	253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr	
-40 -35 -30	
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT	301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala	
-25 -20 -15	
AAA GAA GAA GGG GTA TCT TTG GAT AAA AGA AGT TCA GTG GAG AAG AAG	349
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Ser Ser Val Glu Lys Lys	
-10 -5 1 5	
CCC GAG GGA GTG AAC ACC GGT GCT GGT GAC CGT CAC AAC CTG AAG ACA	397
Pro Glu Gly Val Asn Thr Gly Ala Gly Asp Arg His Asn Leu Lys Thr	
10 15 20	
GAG TGG CCA GAG TTG GTG GGG AAA TCG GTG GAG GAG GCC AAG AAG GTG	445
Glu Trp Pro Glu Leu Val Gly Lys Ser Val Glu Glu Ala Lys Lys Val	
25 30 35	
ATT CTG CAG GAC AAG CCA GAG GCG CAA ATC ATA GTT CTG CCG GTG GGT	493
Ile Leu Gln Asp Lys Pro Glu Ala Gln Ile Ile Val Leu Pro Val Gly	
40 45 50	
ACC ATT GTG ACC ATG GAA TAT CCG ATC GAT CCG GTC CCG CTC TTT GTC	541
Thr Ile Val Thr Met Glu Tyr Arg Ile Asp Arg Val Arg Leu Phe Val	
55 60 65 70	

GAT AAA CTC GAC AAC ATT GCC CAG GTC OCT AGG GTC GGC TAGTGATCTA 590
 Asp Lys Leu Asp Asn Ile Ala Gln Val Pro Arg Val Gly
 75 80

GA 592

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 168 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 -85 -80 -75 -70

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
 -65 -60 -55

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
 -50 -45 -40

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
 -35 -30 -25

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 -20 -15 -10

Ser Leu Asp Lys Arg Ser Ser Val Glu Lys Lys Pro Glu Gly Val Asn
 -5 1 5 10

Thr Gly Ala Gly Asp Arg His Asn Leu Lys Thr Glu Trp Pro Glu Leu
 15 20 25

Val Gly Lys Ser Val Glu Glu Ala Lys Lys Val Ile Leu Gln Asp Lys
 30 35 40

Pro Glu Ala Gln Ile Ile Val Leu Pro Val Gly Thr Ile Val Thr Met
 45 50 55

Glu Tyr Arg Ile Asp Arg Val Arg Leu Phe Val Asp Lys Leu Asp Asn
 60 65 70 75

Ile Ala Gln Val Pro Arg Val Gly
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: barley

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 10..324

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 10..75

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 76..324

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AGC GCC TTG GCC AGT CCT ATT CGT CGA AGC TCA GTG GAG AAG AAG CCC	96
Thr Ala Leu Ala Ser Pro Ile Arg Arg Ser Ser Val Glu Lys Lys Pro	
-5 1 5	
GAG GGA GTG AAC ACC GGT GCT GGT GAC CGT CAC AAC CTG AAG ACA GAG	144
Glu Gly Val Asn Thr Gly Ala Gly Asp Arg His Asn Leu Lys Thr Glu	
10 15 20	
TGG CCA GAG TTG GTG GGG AAA TCG GTG GAG GAG GCC AAG AAG GTG ATT	192
Trp Pro Glu Leu Val Gly Lys Ser Val Glu Glu Ala Lys Lys Val Ile	
25 30 35	
CTG CAG GAC AAG CCA GAG GCG CAA ATC ATA GTT CTG CCG GTG GGT ACC	240
Leu Gln Asp Lys Pro Glu Ala Gln Ile Ile Val Leu Pro Val Gly Thr	
40 45 50 55	
ATT GTG ACC ATG GAA TAT CGG ATC GAT CGC GTC CGC CTC TTT GTC GAT	288
Ile Val Thr Met Glu Tyr Arg Ile Asp Arg Val Arg Leu Phe Val Asp	
60 65 70	
AAA CTC GAC AAC ATT GCC CAG GTC CCT AGG GTC GGC TAGTGATCTA	334
Lys Leu Asp Asn Ile Ala Gln Val Pro Arg Val Gly	
75 80	
GA	336

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 105 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
-22 -20 -15 -10

Ala Ser Pro Ile Arg Arg Ser Ser Val Glu Lys Lys Pro Glu Gly Val
-5 1 5 10

Asn Thr Gly Ala Gly Asp Arg His Asn Leu Lys Thr Glu Trp Pro Glu
 15 20 25

Leu Val Gly Lys Ser Val Glu Glu Ala Lys Lys Val Ile Leu Gln Asp
 30 35 40

Lys Pro Glu Ala Gln Ile Ile Val Leu Pro Val Gly Thr Ile Val Thr
 45 50 55

Met Glu Tyr Arg Ile Asp Arg Val Arg Leu Phe Val Asp Lys Leu Asp
60 65 70

Asn Ile Ala Gln Val Pro Arg Val Gly
75 80

CLAIMS

1. A detergent composition comprising a protease and a reversible protease inhibitor of peptide or protein type, characterized in that the ratio of the dissociation constant to the protease concentration is in the range from 0.006 to 5 6.
2. A composition according to Claim 1, wherein said ratio is in the range from 0.06 to 6.
3. A detergent composition comprising a protease and a reversible protease inhibitor of peptide or protein type, characterized by an dissociation 10 constant in the range from 0.05 to 50 μ M.
4. A composition according to Claim 3, wherein said constant is in the range from 1 to 10 μ M.
5. A composition according to any of Claims 1 - 4, wherein the protease is a serine protease, preferably an alkaline microbial protease or a trypsin-like 15 protease.
6. A composition according to Claim 5, wherein the trypsin-like protease is trypsin or is derived from *Fusarium*.
7. A composition according to Claim 5, wherein the alkaline microbial protease is a subtilisin.

8. A composition according to Claim 7, wherein the inhibitor is a subtilisin inhibitor of family VI or a mutant thereof, in which the amino acid residue at one or more of the positions P6, P5, P4, P3, P2, P1, P'1, P'2, P'3 is substituted with another amino acid residue.
- 5 9. A detergent composition comprising a subtilisin, characterized by further comprising a modified subtilisin inhibitor of Family VI having on or more of the following amino acid substitutions at the indicated position:
- P6: Ala, Glu or Lys,
P5: Gly, Val, Leu or Pro
10 P4: Val, Pro, Trp, Ser, Glu or Arg,
P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp,
P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala,
P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys, Ala,
P'1: Gln, Ser, Thr, Ile, Lys or Pro,
15 P'2: Val, Glu, Arg, Pro or Trp
P'3: Glu, Gln, Asn, Val, Phe or Tyr.
10. A composition according to Claim 8 or 9, wherein the inhibitor is a modified barley subtilisin inhibitor CI-1 or CI-2, potato subtilisin inhibitor (PSI), Eglin B or C, tomato subtilisin inhibitor or *Vicia* subtilisin inhibitor (VSI).
- 20 11. A composition according to any of Claims 7 - 10, wherein the subtilisin is derived from *Bacillus* and is preferably subtilisin Novo, subtilisin Carlsberg, BPN', subtilisin 309, subtilisin 147 or subtilisin 168.
12. A composition according to any preceding claim, wherein the molar ratio of inhibitor reactive site to protease active site is above 0.6, preferably 1-10.
- 25 13. A composition according to any preceding claim, wherein the amount of protease is 0.2-40 μ M, preferably 1-20 μ M.

14. An aqueous liquid detergent composition according to any preceding claim.
15. A detergent composition comprising a protease and a reversible protease inhibitor, characterized in that the degree of protease inhibition in the
5 detergent is at least 60%, and the degree of protease inhibition in a 1% detergent solution in water is below 10%.
16. A detergent additive comprising protease in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of peptide or protein type whereby the dissociation constant is
10 in the range from 0.05-50 μM .
17. A detergent additive comprising a subtilisin in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a modified subtilisin inhibitor of Family VI having one or more of the following amino acid substitutions at the indicated position:
- 15 P6: Ala, Glu or Lys,
P5: Gly, Val, Leu or Pro
P4: Val, Pro, Trp, Ser, Glu or Arg,
P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp,
P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala,
20 P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys, Ala,
P'1: Gln, Ser, Thr, Ile, Lys or Pro,
P'2: Val, Glu, Arg, Pro or Trp
P'3: Glu, Gln, Asn, Val, Phe or Tyr.
18. A method for stabilizing a protease by incorporation of a protease
25 inhibitor, characterized in that the ratio of the dissociation constant to the protease concentration is in the range from 0.006 to 6.

19. A modified subtilisin inhibitor of Family VI having one or more of the following amino acid substitutions at the indicated position:

- 5 P6: Ala, Glu or Lys,
P5: Gly, Val, Leu or Pro
P4: Val, Pro, Trp, Ser, Glu or Arg,
P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp,
P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala,
P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys, Ala,
10 P'1: Gln, Ser, Thr, Ile, Lys or Pro,
P'2: Val, Glu, Arg, Pro or Trp
P'3: Glu, Gln, Asn, Val, Phe or Tyr.

excluding:

- Eglin B and C substituted with
Ser or Pro at position 44 (P2),
15 Leu, Arg, Phe, Tyr at 45 (P1) or
Glu, Ser or Thr at 46 (P'1),
Eglin C substituted with Arg45, Ser46 and
CI-2 substituted with Tyr, Ala or Lys at 59 (P1).

20. A modified barley subtilisin inhibitor CI-1 or CI-2, potato subtilisin inhibitor (PSI), Eglin B or C, tomato subtilisin inhibitor or *Vicia* subtilisin inhibitor (VSI) according to Claim 19.

21. A recombinant DNA molecule comprising a nucleotide sequence coding for the modified subtilisin inhibitor of Claim 19 or 20.

22. A transformed host organism comprising the DNA of Claim 21.

25 23. A method of producing the inhibitor of Claim 19 or 20, characterized by comprising cultivation of the transformed host organism of Claim 20.

1/3

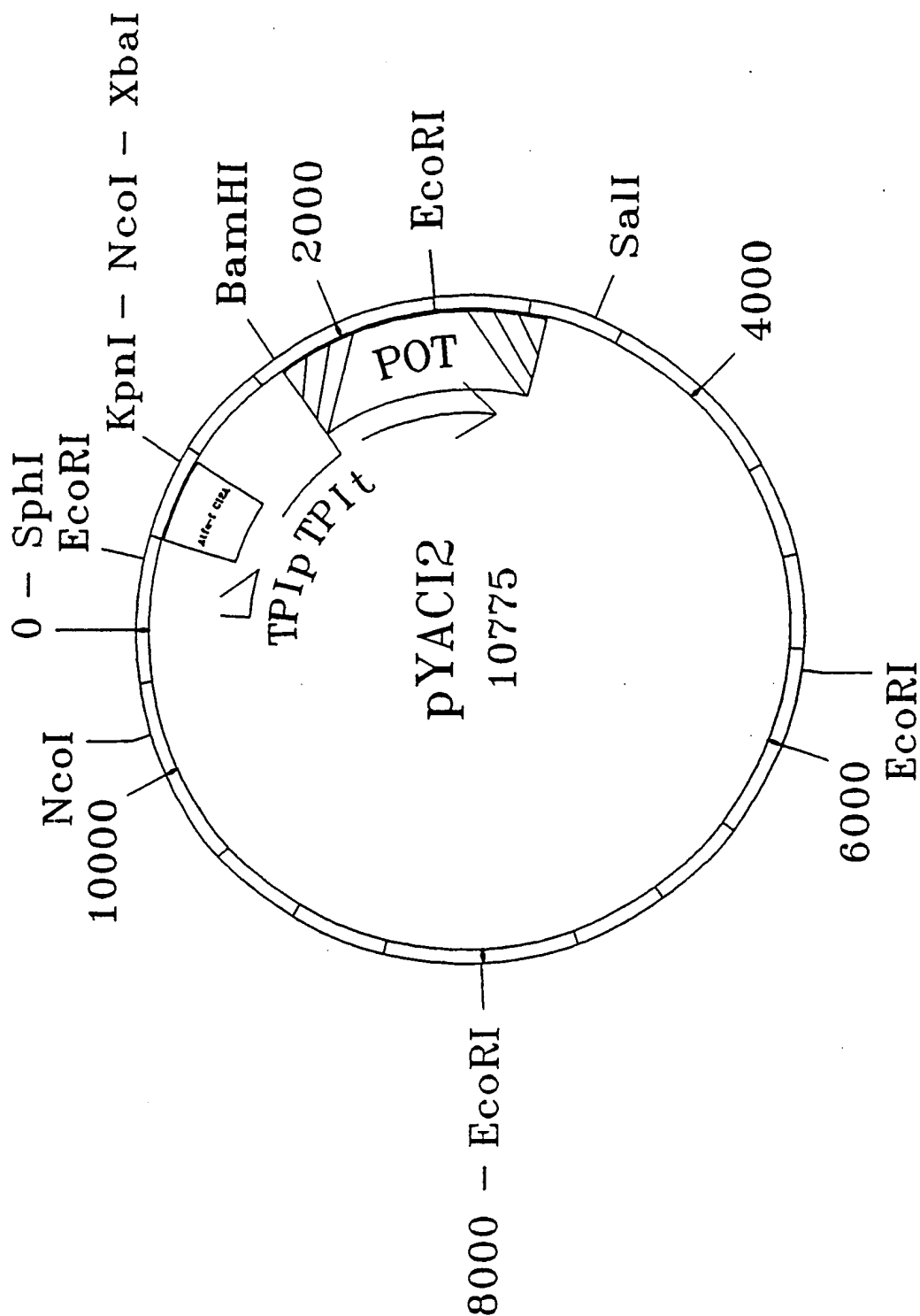


Fig. 1

2/3

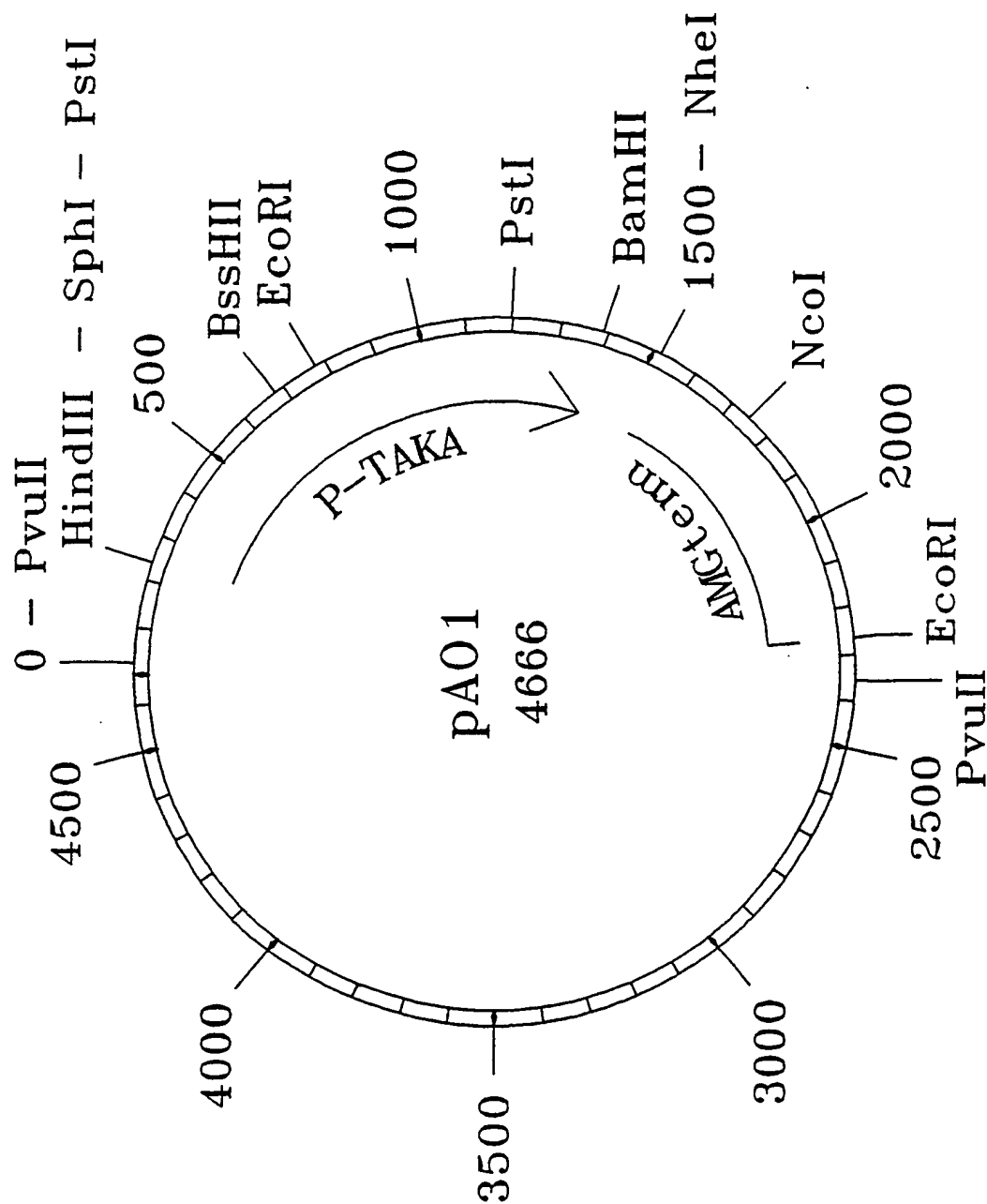


Fig. 2

3/3

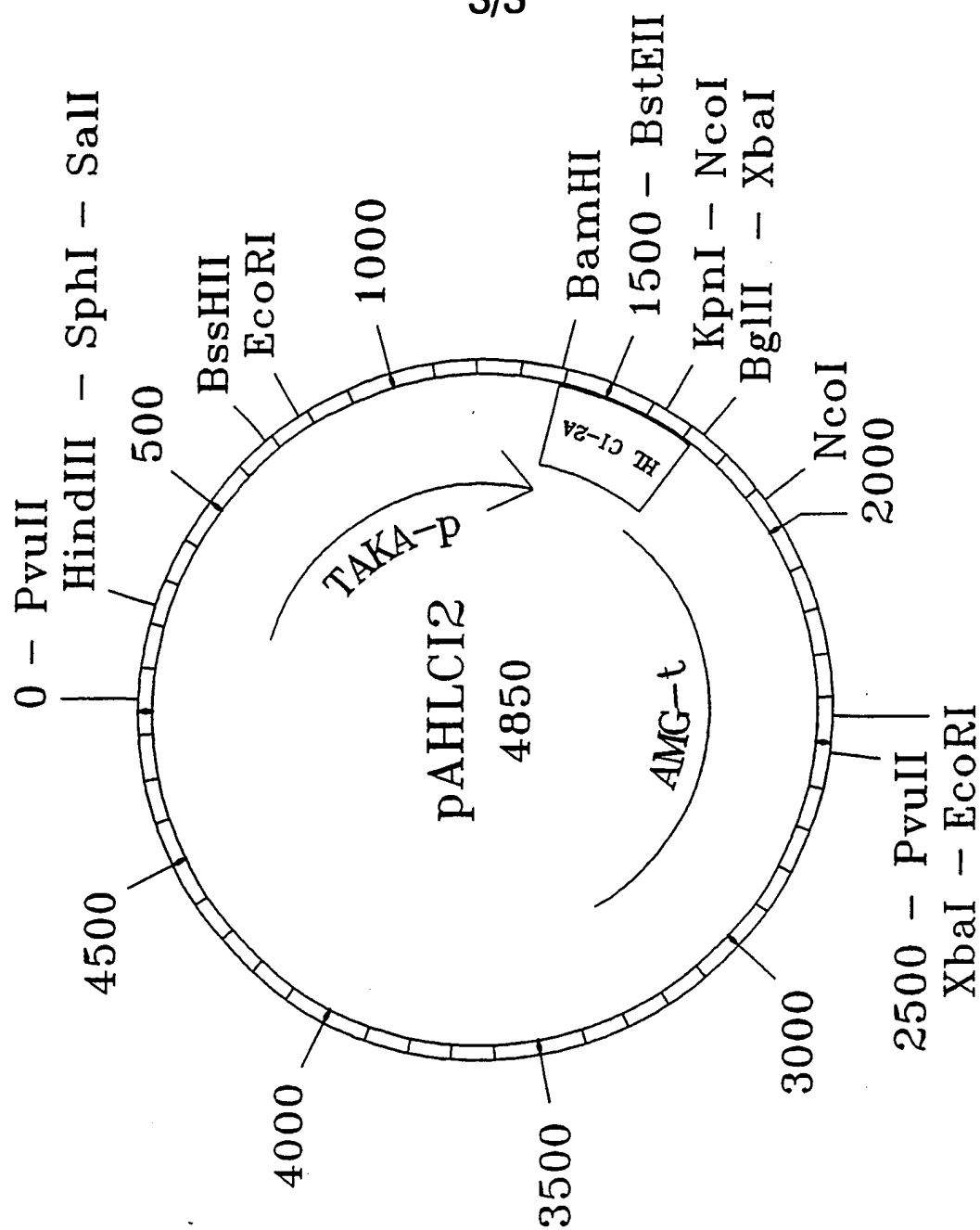
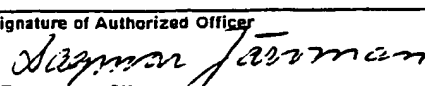
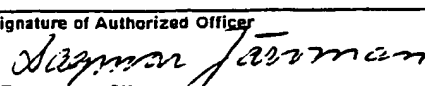
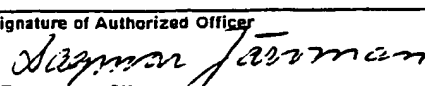


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00279

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 11 D 3/386											
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black; padding: 2px;">Classification System</td> <td style="border-bottom: 1px solid black; padding: 2px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC5</td> <td style="padding: 5px;">C 11 D</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in Fields Searched⁸</div> <p style="padding: 5px;">SE,DK,FI,NO classes as above</p>			Classification System	Classification Symbols	IPC5	C 11 D					
Classification System	Classification Symbols										
IPC5	C 11 D										
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black; padding: 2px;">Category *</th> <th style="width: 70%; border-bottom: 1px solid black; padding: 2px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 20%; border-bottom: 1px solid black; padding: 2px;">Relevant to Claim No.¹³</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">P,X</td> <td style="padding: 5px;">US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4-12; line 46 - column 8, line 1-8; example 2; claim 1-8; abstract <div style="text-align: center;">--</div></td> <td style="vertical-align: top; padding: 5px;">1-7</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) <div style="text-align: center;">-- -----</div></td> <td style="vertical-align: top; padding: 5px;">1-7</td> </tr> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	P,X	US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4-12; line 46 - column 8, line 1-8; example 2; claim 1-8; abstract <div style="text-align: center;">--</div>	1-7	A	Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) <div style="text-align: center;">-- -----</div>	1-7
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P,X	US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4-12; line 46 - column 8, line 1-8; example 2; claim 1-8; abstract <div style="text-align: center;">--</div>	1-7									
A	Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) <div style="text-align: center;">-- -----</div>	1-7									
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>											
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="padding: 5px;">19th December 1991</td> <td style="padding: 5px; text-align: center;">1991 -12- 20</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 2px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 2px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px; text-align: center;">SWEDISH PATENT OFFICE</td> <td style="padding: 5px; text-align: center;">  Dagmar Järvmä </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	19th December 1991	1991 -12- 20	International Searching Authority	Signature of Authorized Officer	SWEDISH PATENT OFFICE	 Dagmar Järvmä	
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report										
19th December 1991	1991 -12- 20										
International Searching Authority	Signature of Authorized Officer										
SWEDISH PATENT OFFICE	 Dagmar Järvmä										

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00279

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 31/10/91
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 5039446	91-08-13	NONE	

